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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : A61K 47/28, 48/00, C07H 21/02, 21/04, C12N 15/00 — A1

(11) International Publication Number: WO 97/14440

(43) International Publication Date: 24 April 1997 (24.04.97)

(21) International Application Number: PCT/US96/16796

(22) International Filing Date: 18 October 1996 (18.10.96)

(30) Priority Data:

60/005,544	19 October 1995 (19.10.95)	US
60/012,037	21 February 1996 (21.02.96)	US

(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.

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(54) Title: CONJUGATION OF C-MYC ANTISENSE OLIGONUCLEOTIDES WITH CHOLESTEROL TO SIGNIFICANTLY ENHANCE THEIR INHIBITORY EFFECT ON NEOINTIMAL HYPERPLASIA

(57) Abstract

Antisense oligonucleotides have recently been proposed as a pharmacological approach to prevent neointimal hyperplasia following balloon angioplasty. This study reports the significant enhancement on in vivo restenosis prevention conferred by the conjugation of cholesterol to a c-myc antisense oligomer when compared to a non conjugated oligonucleotide. The inhibitory potential of the conjugate was increased 2.4-fold, reaching a reduction of intimal hyperplasia of 84.7 % vs. control arteries. Enhancement of hyperplasia prevention may be related to the 2.7-fold increase in oligomer vascular retention conferred by the cholesterol moieties, sufficient to cover a significant portion of the proliferation window that typically follows balloon angioplasty.

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CONJUGATION OF C-MYC ANTISENSE OLIGONUCLEOTIDES WITH CHOLESTEROL TO SIGNIFICANTLY ENHANCE THEIR INHIBITORY EFFECT ON NEOINTIMAL HYPERPLASIA

RELATED APPLICATIONS

This application is based upon provisional applications S.N. 60/005,544 filed October 19, 1995 and S.N. 60/012,037 filed February 21, 1996, both of which concern the conjugation of c-myc antisense oligonucleotides with cholesterol.

BACKGROUND OF THE INVENTION

The ability of antisense oligomers to interact specifically with mRNAs of their corresponding sequences provides a valuable tool for the control of cellular expression. Antisense oligodeoxynucleotides (ODNs) have recently been successfully used as "informational drugs" to prevent smooth muscle cell proliferation in vitro and neointimal hyperplasia after balloon angioplasty. During development of this strategy, other investigators delivered oligonucleotides through the use of a pluronic gel surgically applied on the adventitial layer of the artery to permit sustained and prolonged DNA delivery to the angioplastied vessel. This approach is, however, inapplicable in human clinical angioplasty procedures. The importance of continued local presence of the therapeutic molecule at a sufficient concentration at the site of angioplasty must not be underestimated, since the proliferation window of vascular smooth muscle cell (SMC) has been shown to last up to 14 days following angioplasty. Moreover, the inappropriate delivery profile of numerous other molecules

tried in clinical studies aimed at preventing restenosis may explain in part the failure seen so far in all these pharmacological trials.

In order to optimize their level of cellular incorporation, oligonucleotides have been covalently linked to a variety of hydrophobic groups, such as cholesterol. This lipidic conjugation of oligomers have proven efficient for in vitro inhibition of HIV multiplication. In vitro studies have also documented enhanced cellular uptake in neutrophils and HeLa cells. Furthermore, two to three fold improved oligomers nuclease resistance was associated with cholesterol conjugation in hepatoma cells. Little information, however, is available on the fate of cholesterol-coupled oligonucleotides in vivo. Association of 5'-cholesterol-bearing oligonucleotides with low and high density lipoproteins demonstrated a prolonged plasma half-like in rats, increasing from less than 1 minute to 9-11 minutes.

SUMMARY OF THE INVENTION

In order to apply and optimize the efficacy of ODN prevention of neointimal hyperplasia following balloon angioplasty in a human clinical setting, we demonstrate that coupling all oligonucleotide to cholesterol significantly enhances the ODN efficacy to prevent neointimal hyperplasia following balloon angioplasty. This enhanced inhibitory effect of cholesterol conjugation is demonstrated with c-myc mRNA targeting but may also be applied to any other DNA oligonucleotide targeting mRNA switched in smooth muscle cell proliferation such as c-myb, PCNA and others. This invention relies on the demonstration that cholesterol-conjugation of the oligonucleotide significantly increases the ODN retention in vascular tissue following in vivo transfection. Accordingly, the purpose

of this invention evaluates, first, in vitro, the effect of conjugation of c-myc antisense phosphorothioate oligomers with cholesterol on ODNs pharmacokinetics properties and effect on smooth muscle cell proliferation. Then, in vivo, following direct arterial transfection, evaluate the long term outcome of transfected cholesterol-antisense oligomers and their effect on neointimal hyperplasia formation.

DESCRIPTION OF THE DRAWINGS

Figure 1: Sense, antisense and scrambled oligonucleotides conjugated with cholesterol moieties.

Figure 2: In vitro inhibition percentage of smooth muscle cells proliferation following incubation with 5 μM or 10 μM of sense or antisense oligonucleotides either alone or conjugated with cholesterol (n = 4 for each group).

Figure 3: Sucrose gradient distribution of fractions derived from in vitro transfected SMCs with 10 μM of either cholesterol-conjugated or unconjugated antisense oligonucleotides. Each experiment was repeated in triplicates producing similar results. Nucleotidase activity of each fraction is presented as a ratio of the highest value obtained within the sucrose gradient. For the comparison between ^{35}S-labeled cholesterol-conjugated and unconjugated ODNs association with gradient fractions, ^{35}S radioactivity quantified was standardized for a same amount of evaluated 5'-nucleotidase activity.

Figure 4: Intra-arterial distribution of ^{35}S-labeled unconjugated and conjugated ODNs (80 μM) transfected into isolated rabbit carotid arteries (1 cm, 20 mg of tissue) for 30 minutes. n = 3 for each group.

Figure 5: Photomicrograph of histological section derived from rabbit carotid artery treated with 80 μM of fluoresceine-conjugated cholesterol antisense ODNs for 30 minutes in an isolated arterial segment (1 cm, 20 mg of tissue). "IEL" indicates internal elastic lamina and "M" indicates medial layer. Original magnification x 1000.

Figure 6: ^{35}S-ODNs retention into normal carotid arteries was evaluated at 3, 6, 12, 24, 48, 72 and 168 hours following transfection with c-myc antisense ODNs (80 μM, 30 min), either alone or conjugated with cholesterol (n = 3 for each group). The retained ^{35}-S labeled oligomers were evaluated by radioactivity quantification. Post-transfection at 0 hours, corresponding to 30 minutes of arterial exposure to oligonucleotides, was considered as "100% incorporation".

Figure 7: ^{35}S-labeled oligonucleotides extraction from normal arterial segments exposed to oligomers (80 μM, 30 minutes). Each experiment was repeated in triplicata, producing similar results.

Figure 8-11: Fig. 8-unconjugated sense ODNs, Fig. 9 = unconjugated antisense ODNs, Fig. 10 = cholesterol-conjugated sense ODNs, Fig. 11-cholesterol-conjugated antisense ODNs. Photomicrographs of histological sections derived from rabbit doubled injured carotid arteries treated with 80 μM of oligonucleotides. "IEL" indicates internal elastic lamina and "N" indicates neointimal hyperplasia. Original magnification x 1000. (Hematoxylin and Eosin stain).

Figure 12: Ratio of neointima/media areas of double injured arterial segments treated with control (100 μl of NaCl 0.9%), 80 μM of sense ODNs, antisense ODNs, cholesterol-conjugated sense ODNs, cholesterol-conjugated antisense ODNs or cholesterol-conjugated scrambled ODNs. Intimal/medial areas were evaluated by computer analysis on histological section derived from transfected arteries two weeks following the second injury and compound transfection (n = 6 for each group).

DETAILED DESCRIPTION OF THE INVENTION

Material and Methods

Cholesterol conjugation of oligonucleotides

Phosphorothioate antisense ODNs, 15 mer in length, complementary to c-myc (5'-CAC GTT GAG GGG CAT-3'), the corresponding sense sequence (5'-ATG CCC CAC AAC GTG-3') and scrambled sequence (5'-CAC TGT TAG GGG AAG-3') were synthesized on a 392 DNA/RNA synthesizer following standard procedure (Applied Biosystems). This target was selected to inhibit smooth muscle cell proliferation in vitro. Conjugation of oligomers with cholesterol was achieved with 3'-cholesterol-VN CPG (Clontech), a virtual nucleotide (VN) glass reagent that introduces a cholesterol label to the 3' terminus of an oligonucleotide via solid-phase synthesis. When incorporated into an oligonucleotide, 3'-cholesterol-VN CPG introduces a deoxyribose sugar moiety that mimics a natural nucleotide base unit. As a result, the identical sugar-phosphate DNA backbone is maintained: stereochemical definition, enantiomeric

purity, conformational rigidity, and internucleotide phosphate distance are all conserved (Figure 1).

When ODN synthesis was completed, radiolabeled oligomers were removed from the column with 30% NH^4OH (1 hour at room temperature), and then deprotected for 8 hours at 60°C. Oligomers were purified and detritylated with oligonucleotide purification cartridges (Applied Biosystems), and then lyophilized with a centrifugal evaporator (Savant SpeedVac). Prior to transfection, oligomer concentration was assessed by spectrophotometry at 260 nm. Following ethidium bromide staining (0.3 μg/μl), the comparison of oligomer migration with standard DNA, under UV light, showed only 15 mer ODNs in the final preparation to be transfected.

<u>^{35}S labelling of oligomers</u>

In order to permit detection and quantification of transfected oligonucleotides, these oligomers were internally radiolabeled by replacing the standard sulfurizing step of base 3, 6, and 9 with a radiolabelling ^{35}S solution (200 μl of TEDT/Acetonitrile + 5μl of ^{35}S [0.373 mCi]).

ODN purification was performed as described above, with oligonucleotide purification cartridges. ^{35}S radioactivity quantification was evaluated with a Beckman LS 8100 scintillation counter, and ^{35}S-ODN integrity was verified, as described above, by electrophoresis on a 20% urea-polyacrylamid gel. Furthermore, radioactivity emission of ^{35}S-ODN was trapped on a film (Kodak) for several days (in darkness at -80°C), and oligomer location was indicated by a dark band. Only 15 mer oligomers were detected on these radiographic films.

Assessment of smooth muscle cell proliferation index in vitro

Primary cultures of rabbit vascular smooth muscle cells were obtained from explants of New-Zealand rabbit thoracic aorta which were surgically removed, cleaned and kept at 37°C under 5% CO_2 in 10 ml of D-MEM medium supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, penicillin (100 U/ml) and streptomycine (100 U/ml). Cells from passage 3 to 5 were used in this study.

Inhibitory effect of oligonucleotides on smooth muscle cell proliferation was assessed by thymidine incorporation index of untreated cells and also cells treated with 5 μM or 10 μM c-myc antisense (cholesterol-conjugated or not), and c-myc sense (cholesterol-conjugated or not). Cells were plated at a density of 5000 cells/cm^2, and allowed to recover 24 hours in D-MEM (Gibco-BRL) supplemented with 10% FBS. Cells were synchronized to a same state of proliferation by supplementing the medium with 0.5% FBS for 48 h. Increase of serum content to 10% FBS activated cell proliferation and oligonucleotides were added to the medium. After 12 hours of incubation, ^3H thymidine was added (5 uCi/ml) and 12 hours later the medium was removed. Cells were washed with cold phosphate buffered saline, fixed for 10 minutes in ethanol:acetic acid (3:1), washed with distilled water and incubated for 15 minutes in ice-cold 0.5N perchloric acid. Following a rapid wash in 0.5N perchloric acid, cells were incubated for 20 minutes at 80°C in 0.5N perchloric acid, and evaluated in a scintillation counter for ^3H radioactivity emission. All experiments were performed at least in triplicates.

Sucrose gradient of transfected SMCs

To assess potential intracellular distribution sites of cholesterol-conjugated ODNs we evaluated the association of ^{35}S-labeled oligomers with cellular membranes. Smooth muscle cells were synchronized to a quiescent state by supplementing the D-MEM medium with 0.5% FBS for 48 h. Cells were stimulated to proliferate by changing the medium to 10% FBS D-MEM and unconjugated or cholesterol-conjugated antisense oligonucleotides (10 μM) were then added to the medium. After 24 hours of incubation, cells were removed from culture plates with 4 ml of trypsine and neutralized with 10 ml of cold PBS. Cells were then centrifugated at 1900 rpm for 7 minutes (4^oC), and the resulting pellet was resuspended in a solution of 200 mM sucrose:5 mM $NaPO_4$ (pH = 7.5). Smooth muscle cells plasmic membranes were permanently destabilized by applying a high power ultrasound (2x15 seconds) resulting in intracellular components release. By centrifugating this solution at 800 g x 5 minutes, the few cells maintaining their integrity were pulled apart in the pellet. The supernatant containing the intracellular components and plasmic membranes from destabilized cells was then deposed on a 20-54% sucrose gradient prepared with 5mM of $NaPO_4$ (pH = 7.5). An ultracentrifugation at 38000 rpm for 16 hours (3^oC) allowed cell particles to sedimentate through the gradient in separate zones characterized by their sedimentation rate. Gradient fractions of 250 μl were collected, each fraction being examined for associated ^{35}S labeled antisense oligonucleotides and 5'-Nucleotidase activity indicating the presence of membranes. ^{35}S labeled ODNs (100 μl of sample) were quantified on a Beckman LS 8100 scintillation counter.

For 5'-nucleotidase activity quantification, fraction samples of 100 μl were mixed with 650 μl of reactional solution (50 mM of glycine, pH = 9;

0.4 mM of $MgCl_2$; 0.16 mM of 5'AMP) and incubated for 30 minutes at 37°C. The reaction was stopped with successive addition of 39.5 µl of $ZnSO_4$ 750 mM and 474 µl of $Ba(OH)_2$ 100 mM (final concentration of 37.5 mM) at 4°C. Following a centrifugation at 14,000 rpm for 5 minutes, 5' nucleotidase activity was quantified on a spectrophotometer at 260 nm.

In vivo arterial ODN transfection: pharmacokinetic studies

New Zealand rabbits male or female (2 Kg) were intramusculary sedated with xylazine (2 mg/Kg) and anesthetized with ketamine (100 mg/Kg) prior to surgical exposure of left carotid artery. Additional doses of anesthesia were given intramusculary throughout the experiment, as needed. Segments (10 mm, corresponding approximately to 20 mg of tissue) of carotids were transiently isolated by temporary ligatures and rinsed with 0.9% sodium chloride via a cannula until there was no more visible evidence of blood components. Carotid arteries (n=57) were transfected with 80 µM of c-myc ^{35}S labeled antisense ODNs in a 1 cm portion either alone (n=27) or conjugated to cholesterol (n=30) for a period of 30 minutes. The volume infused was 100 µl, and no visible loss of volume was noted throughout the incubation period. Following transfection, the treated segments were rinsed with 0.9% sodium chloride (3 x 100 µl) and upon cannula removal, the arteriotomy site was repaired with microsutures (100 microns nylon monofilament, Pike Surgical Inc. Calgary, Canada), restoring normal blood flow. The neck wound was then closed and no adverse neurological or vascular effects were observed in any animal undergoing this procedure. Following surgery, the rabbits were allowed to recuperate, housed in approved facilities with free access to rabbit chow and water, and exposed to a 12-hour light dark cycle.

To assess arterial retention of transfected oligomers, rabbits were sacrificed at 3, 6, 12, 24, 48, 72 and 168 hours post-transfection. Both carotid segments, from the same animal, were removed as the right common carotid artery was considered as an internal control for ^{35}S contamination. Incorporation and retention levels of 35-S ODNs were evaluated by scintillation counting following exposure of treated segments to liquid nitrogen, thus releasing the incorporated oligomers.

Physical integrity of oligos

Oligonucleotide physical integrity was assessed following oligomers extraction from the arterial segments at 72 hours and 168 hours post-transfection. The transfected segments were removed surgically and crushed into fine powder following exposure to liquid nitrogen. ^{35}S-labeled ODN were then extracted from the vascular cells and were separated from cellular DNA by further extraction with an equal volume of phenol (pH = 6.8). Oligos recovered in the aqueous phase were then reextracted with a 50:50 mixture of phenol and equilibrated chloroform:isoamyl (24:1), followed by an equal volume of chloroform:isoamyl. The recovered ^{35}S-labeled ODN were desalted with oligonucleotide purification cartridges (Applied Biosystems), the cartridges being rinsed with 5 ml of acetonitrile (99%) of 5 ml of TEAA 2M prior to oligo application. Oligomers retained by the cartridges were rinsed with 10 ml of H_2O and were eluted with 20% of acetonitrile, llyophilized and then solubilized in deionized water. Recovery of ^{35}S-labeled oligos applied on the cartridge varied between 80% and 90%. Oligomers physical integrity was verified by electrophoresis on a 20% urea-polyacrylamid gel, oligos migraton being compared to standard DNA following ethidium bromide coloration. Furthermore, visualization by autoradiography indicated that

the totality of ^{35}S radioactivity recovered from the transfected arteries was coupled to the 15 mer oligos.

Arterial distribution of ODNs

Oligos biodistribution in the vessel wall was evaluated by dot counting on histological sections derived from transfected arteries. Representative sections of carotid arteries were fixed in 4% paraformalin and embedded in paraffin, and 5-μm sections were cut. ^{35}S radioactivity emission from the vascular cells was trapped by photographic emulsion (in darkness at $4^{o}C$, Kodak autoradiography emulsion). Following development (2.5 minutes in Kodak developer) and fixation (5 minutes in Kodak fixer), ^{35}S-labeled oligomers were visualized as black dots. Histological sections were stained with hematoxylin/eosin (for cells identification) and examined under light microscopy for oligomers transfection into the arterial wall. The average dots counted in the different layers of the carotid artery were extrapolated for the segment treated. Oligomers distribution percentage in a specific layer was then compared to the entire segment. A high correlation factor ($r^2 = 0.96$) was found between dot and scintillation counting.

Thereafter, in vivo intracellular distribution of oligomers was visualized by conjugation with a fluoreseine-isothyocyanate molecule (FITC), introduced at the 5' end of c-myc cholesterol-antisense ODNs via standard automated synthesis (Applied Biosystems). Oligomer purification and detritylation was performed, as described above, with ODN purification cartridges. Oligomer integrity was verified by electrophoresis on a 20% urea-polyacrylamid gel, and oligomer migration was compared to standard DNA under UV light. The totality of fluoresceine molecules detected were

coupled to 15 mer oligomers. Fluorescine-conjugated antisense ODNs (80 μM) were locally transfected into rabbit carotid arteries for 30 minutes and histological sections were derived from these carotid arteries. Representative sections were fixed in 4% paraformalin, embedded in paraffin.

Neointimal hyperplasia inhibition

A total of 36 New Zealand white rabbit carotid arteries were inured with a 2.5 mm balloon catheter serially inflated for 1 minute to 4, 6, 8 and 10 atm with gentle traction allowing 45 seconds between inflations. Two weeks later, a second injury was imposed at the same arterial site which was then transfected in a 1 cm portion with 80 μM (100 μL of volume injected) of either c-myc antisense alone or conjugated with cholesterol, with c-myc sense alone or conjugated with cholesterol, with cholesterol-conjugated scrambled, or with 100 μL of NaCl 0.9% as control. Intimal/medial areas were evaluated by computer analysis on histological sections derived from transfected arteries two weeks following the second injury and transfection procedure.

Statistical analysis

All values are expressed as mean ± SEM. Analysis of Student's t test was used to determine significant differences between groups. A value of $P < 0.05$ was considered significant.

In vitro studies

In order to determine the potential usefulness of conjugating c-myc antisense oligomers with cholesterol in the prevention of neointimal hyperplasia formation, we first studied the effect of each type of oligomer, sense and antisense, conjugated or not with cholesterol, on primary cultures of rabbit vascular smooth muscle cells. Consistent with previous studies, unconjugated antisense oligomers manifested a dose-response effect on SMCs growth inhibition, reaching a miximal inhibition potential of 72.8$\pm$2.9% at 10 μM (P=0.02 vs 58.0$\pm$4.4% at 5 μM) (Fig. 2). Interestingly, at a dose of 10 μM conjugated-antisense oligonucleotides manifested significant advantage over unconjugated oligomers as their antiproliferative potential increased to 91.3$\pm$2.1% of control (P=0.005 vs. unconjugated oligomers). Although cholesterol-conjugated sense sequences presented a discrete inhibitory effect at both 5μM and 10 μM concentrations, this effect did not reach 20% inhibition (P=0.0001 vs. corresponding antisense). Importantly, all treated cells preserved their normal morphology in presence of conjugated as well as unconjugated oligomers. Also, cholesterol alone, added to our cells cultures, did not affect SMC appearance or proliferation rate (data not shown).

In addition, to further characterize the enhanced antiproliferative potential of cholesterol-conjugated antisense ODNs, we sought to examine intracellular distribution sites of transfected radiolabelled oligomers. Following SMCs particle sedimentation on sucrose gradient we observed a well defined peak for both gradients performed on conjugated and unconjugated-ODN transfected cells (Fig. 3). Radioactive intensity of this peak was evaluated at 2484 cpm for cholesterol-conjugated ODNs and at 863 cpm for unconjugated oligomers. This represented a 2.9-times

increase in cholesterol-conjugated ODN uptake and retention level when compared to unconjugated ODNs (P = 0.001). The significant increase observed supports the concept that cholesterol conjugation of oligomers may favorably influence the pharmacokinetic properties of these therapeutic molecules. We sought to further examine the nature of cellular fractions corresponding with oligomers presence, as we evaluated membranal fractions by dosage of 5'-nucleotidase activity. Measurement of this enzyme activity revealed high levels of 5'-nucleotidase in fractions where oligomers were the most concentrated (Fig. 3), suggesting that oligomers preferentially locate in membrane components of SMCs.

In vivo studies

In order to efficiently prevent in vivo neointimal hyperplasia formation following balloon angioplasty, the active molecule must be efficaciously delivered to the vessel wall and preferably impose its presence to the injured area as long as possible during the proliferation window that occurs following the trauma. Accordingly, we examined in this series of experiments the level of ODNs successfully transfected in the target vessel wall, its tissue distribution, and vascular retention.

At 0 hours post-transfection, cholesterol-conjugated antisense ODNs were more efficiently transfected into injured carotid arteries when compared to unconjugated ODNs and reached, respectively, 40.3$\pm$0.4% and 28.2$\pm$0.4% (P = 0.03) of available oligomer exposed to the vessel wall, while incorporation in normal arteries was of similar extent for both antisense formulations (29.2$\pm$1.3% for unconjugated antisense, 30.1$\pm$1.7% for cholesterol-conjugated antisense, P = NS). With respect to oligomer distribution (Fig. 4), we observed that both conjugated and

unconjugated oligomers were mainly located in the medial layer of transfected arterial segments (unconjugated ODN:83.0±0.9% of total ODN incorporated, cholesterol-conjugated ODN: 85.1±0.4% of total ODN incorporated, P = NS). In order to further characterize oligomer distribution and to assess their cellular incorporation, we conjugated short DNA sequences with fluoresceine-cholesterol molecules. As seen on Figure 5, transfected cholesterol oligomers were mainly located in the medial layer with preferential nuclear accumulation. Also, a high level of transfected cells were present in this layer, being suggested by the finding that over 85% of cells showed fluoresceine activity.

With respect to vascular retention (Fig. 6), we observed that cholesterol conjugation of DNA conferred a significant advantage over unconjugated DNA, in normal as in injured arteries, increasing retention levels at 7 days by 2.7-fold, with halftime retention enhanced from 2.2 hours for unconjugated ODNs to 5.9 for cholesterol-conjugated ODNs. At one week, 24.2±1.3% of the transfected conjugated ODN was still present in the vessel wall, compared to 6.2±1.1% (P=0.001) of the unconjugated ODN. Furthermore, DNA extraction from transfected carotid arteries revealed that oligomers remained intact, respectively for unconjugated and cholesterol-conjugated ODNs, for at least 72 hours and 168 hours post-transfection (Fig. 7).

Neointimal hyperplasia inhibition

In order to assess the potential advantage of DNA conjugation with cholesterol on in vivo neointimal hyperplasia inhibition, we examined the efficiency of the five different oligomer formulations to prevent neointimal hyperplasia (Fig. 8-11). Following arterial double balloon injury, maximal

ratio of intimal/medial area was observed in the control group (0.79±0.04), unconjugated sense ODNs group (0.72±0.05, P=NS vs. control) (Fig. 8) and cholesterol-conjugated sense ODNs group (0.72±0.04, P=NS vs. control) (Fig. 10). Although unconjugated antisense ODNs and cholesterol-conjugated scrambled ODNs reduced intimal/medial ratio area respectively to 0.47±0.02 (P=0.001 vs. unconjugated sense ODNs) (Fig. 9) and 0.54±0.02 (P=0.002 vs. cholesterol-conjugated sense ODNs), cholesterol-conjugated antisense ODNs were far more effective, the intimal/medial area ratio being estimated at 0.11±0.01 (P=0.0001 vs. cholesterol-conjugated sense ODNs, cholesterol-conjugated scrambled ODN and control) (Fig. 11). Thus, arterial neointimal hyperplasia was specifically reduced by 34.7% following transfection of unconjugated antisense c-myc ODNs, and by 84.7% following transfection of cholesterol-conjugated antisense ODNs (Fig. 12). Not only was the antiproliferative potential of antisense ODNs increased 2.4 times following oligomers conjugation with cholesterol, but this effect was homogeneously found over the entire neointimal areas.

Antisense oligonucleotide transfection has recently emerged as a potential therapeutic pathway to prevent neointimal proliferation component of restenosis. Several studies have demonstrated that antisense directed against c-myc mRNA not only inhibits smooth muscle cell proliferation but also smooth muscle cell migration, which constitutes another critical component of smooth muscle cell response to vascular injury and, presumably, therefore of the restenosis process. However, the development of antisense oligonucleotide therapy has not been as simple as first believed, and many critical issues have been highlighted. These include concerns about cellular uptake, sequence-specific and non-sequence-specific biological effects, pharmacokinetics and

pharmacodynamics. Key elements of antisense strategy's success may rely on high transfection efficiency but also, importantly, on sufficient retention of this therapeutic molecule during the proliferation window of SMCs following angioplasty, which occurs mostly during the first week.

This is the first invention to report the perceived advantage of conjugating cholesterol to a therapeutic molecule in arterial application. This approach permitted, potentially through advantageous pharmacokinetic properties of the conjugate, to significantly improve the efficiency to inhibit in vitro smooth muscle cells proliferation and in vivo neointimal hyperplasia formation.

In vitro, conjugation of antisense oligonucleotides with cholesterol moieties was extremely beneficial for oligomers effect, since their inhibitory potential reached 91.3% at a dose of 10 μM. This enhanced antiproliferative effect is correlated with increased intracellular levels of these therapeutic molecules, our study demonstrating that cholesterol-conjugated c-myc antisense ODNs are associated at a level with SMCs membrane components at a level 2.9 times higher than that seen with unconjugated oligomers.

Although the precise mechanisms responsible for inhibition of protein expression in eukariotic cells are not precisely known, intracellular trafficking and distribution of oligonucleotides determine the extent to which an "internalized" oligonucleotide is available to interact directly with its biological targets.

Once internalized, ^{35}S-labeled phosphorothioates oligonucleotides were shown to accumulate in vesicular structures and in the nucleus but

significantly less in the cytoplasm. Consistent with this invention, conjugated as well as unconjugated oligonucleotides entering in vivo SMCs migrate rapidly into the nucleus. It appears that cholesterol-conjugated ODNs are anchored to cellular membranes via cholesterol moieties, rendering oligomers available in the cytoplasmic and/or the nucleoplasm compartment. This is also consistent with our in vivo findings where cholesterol-conjugated oligomers incorporation potential was superior to unconjugated oligomers in injured arteries, suggesting a different intracellular compartmentalization and/or hybridization to corresponding mRNA, the latest being generally available 30 minutes following cell stimulation. Moreover, cholesteryl group attachment at the 3'-terminus of DNA is known to confer an enhance resistance to cellular nucleases and may increase the stability of mRNA/antisense oligomer complexe, thus enhancing antisense ODNs availability within the cell which in vivo could translate as increased vascular retention and enhanced antiproliferative effect on neointimal hyperplasia.

Interestingly, we notice that in vivo cholesterol-conjugated antisense oligomers were retained in vascular cells at a level 2.7 times superior in comparison to unconjugated oligomers. Moreover, we observed a sustained retention of the conjugate's physical integrity, even at 1 week, supporting the hypothesis that the conjugate may still be active, with possibly a higher resistance to nucleases. Thus, an increase in ODN's in vivo stability may provide longer duration of action of these molecules, leading to a decrease in the frequency of administration, ensuring a higher vessel to appropriately targeted antisense oligonucleotides may permanently inhibit the restenosis process.

Single transcatheter and adventitial administration of unconjugated ODNs in vivo have been reported to reduce restenosis by others. This invention suggests, however, that conjugation of ODNs with cholesterol will lead to a significantly superior inhibition of neointimal formation (84.7% with conjugated ODNs vs 36.7% with unconjugated ODNs). Although the presence of the four contiguous guanines has been shown to manifest unusual non-sequence-specific effects, here, the 2.4-fold enhancement of hyperplasia inhibition by cholesterol-conjugated antisenses is highly specific and is not dependant upon the presence of 4-G sequence, as shown by the low inhibition percentage (24.2%) of cholesterol-conjugated scrambled 4-G sequence. Also, it seems unlikely that differences in inhibitory potential of antisense and sense sequences may be due to variations in cellular uptake efficacy, since it has been established by other studies that pharmacokinetic and pharmacodynamic properties of phosphorothioate-oligonucleotides are largely independent of base composition.

For many in vivo applications, oligonucleotides must be administered repeatedly in order to obtain the desired biological effects, partly because oligonucleotides are rapidly eliminated, and also because the target gene products have long half-lives. It appears reasonable to infer that if antisense therapy is to be effective in inhibiting the SMCs proliferation process, the antisense molecules should be actively available in the injured region for at least the duration of the proliferative window period. Thus, by improving oligomer phamacokinetics following 3'-end conjugation with cholesterol, the need for repeated oligomer delivery may be eliminated. Local intravascular delivery of cholesterol-conjugated antisense oligonucleotides into double injured rabbit carotid arteries appears to have an increased potential therapeutic effect over non conjugated-

oligonucleotides. Although protection of oligonucleotides from nucleases digestion, and maintaining intracellular high levels of antisense oligonucleotides, represent potential explanation for this effect, it is not excluded that cholesterol-conjugation may enhance binding affinities of ODNs.

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Our invention demonstrates, that conjugation of c-myc antisense oligodeoxynucleotides with cholesterol moieties increases in vitro ODN uptake/retention levels in vascular smooth muscle cells, alters intracellular ODN distribution, and consequently increases ODN efficiency to inhibit SMC growth. In vivo, following transfection to the vessel wall, this conjugate is retained at levels significantly higher at one week when compared to unconjugated ODNs, with a preserved physical integrity. Importantly, conjugation of c-myc antisense oligodeoxynucleotides with cholesterol group confers a significant advantage over unconjugated ODNs in specifically inhibiting neointimal hyperplasia formation in vivo. These findings raise the possibility of multiple therapeutic opportunities and also the development of cholesterol-conjugated antisense olionucleotides into therapeutically viable drugs for the treatment of restenosis. This finding is important in view of the multiple failed attempts in past clinical trial of restenosis prevention. Such trials may benefit from the optimization of the molecule pharmacokinetic properties prior to entering the clinical arena.

Naturally, as with all inventions, this invention is not limited to the present specification, but rather by the claims and their equivalents.

WHAT IS CLAIMED IS:

1. A method of inhibiting smooth muscle cell proliferation by treating a part of the vasculature with a cholesterol conjugated c-myc antisense ODN.

2. A method of inhibiting smooth muscle cell proliferation by treating a part of the vasculature with a cholesterol conjugated antisense oligomer.

3. The inhibition of restenosis or neointinal hyperplasia through treatment of a vascular lumen with cholesterol conjugated c-myc antisense oligomer.

4. The inhibition of restenosis or neointimal hyperplasis inhibition in a vascular lumen by using oligodeoxynucleotides to prevent smooth muscle proliferation conjugated with a cholesterol moiety.

5. A method of increasing vascular tissue retention of antisense oligomer following transfection in vascular tissue by conjugating said antisense with a cholesterol moiety.

6. A method of increasing vascular tissue retention of an oligodeoxnucleotide able to prevent smooth muscle cell proliferation by conjugation of said oligodeoxynucleotide with a cholesterol moiety.

FIGURE 1

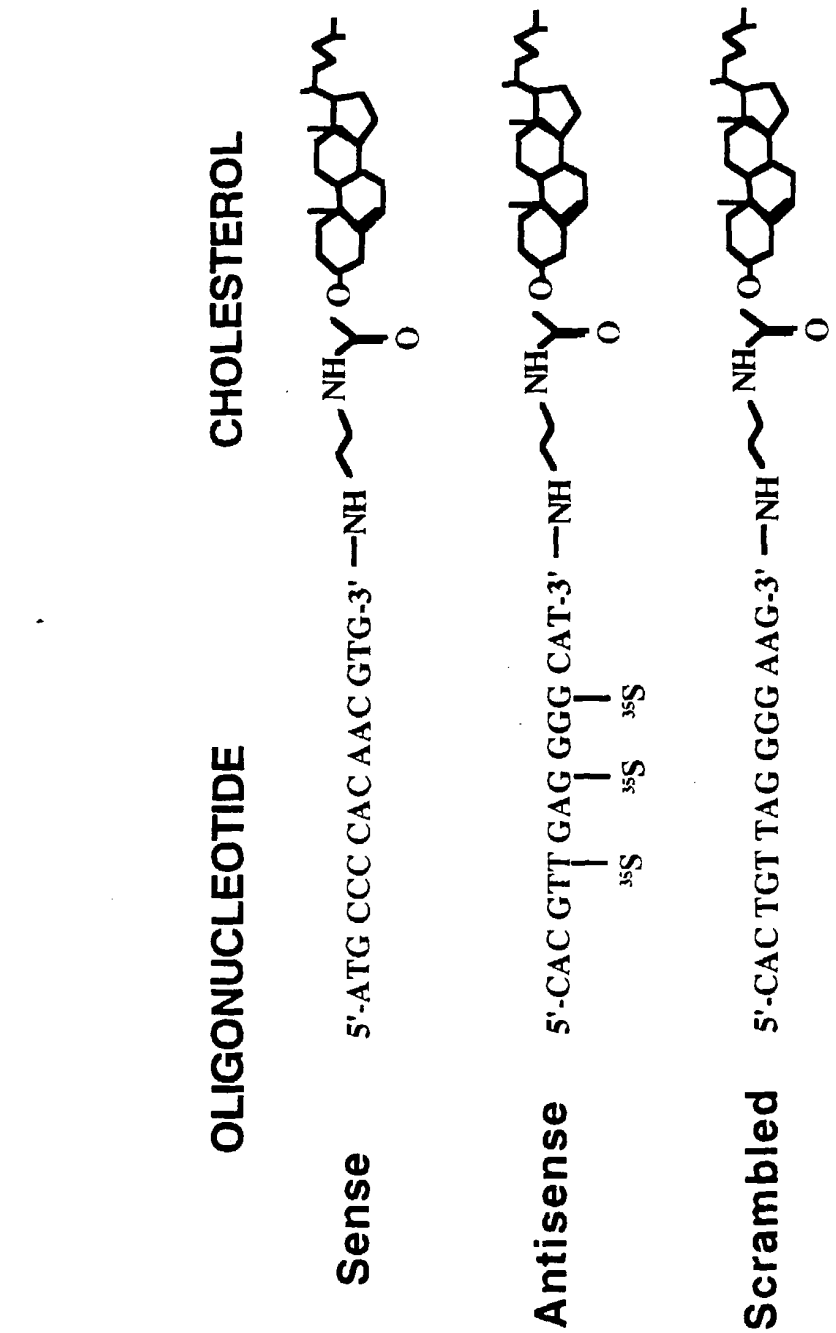

OLIGONUCLEOTIDE
CHOLESTEROL
Sense
5'-ATG CCC CAC AAC GTG-3'
Antisense
5'-CAC GTT GAG GGG CAT-3'
35S
35S
35S
Scrambled
5'-CAC TGT TAG GGG AAG-3'
NH
O

FIGURE 2

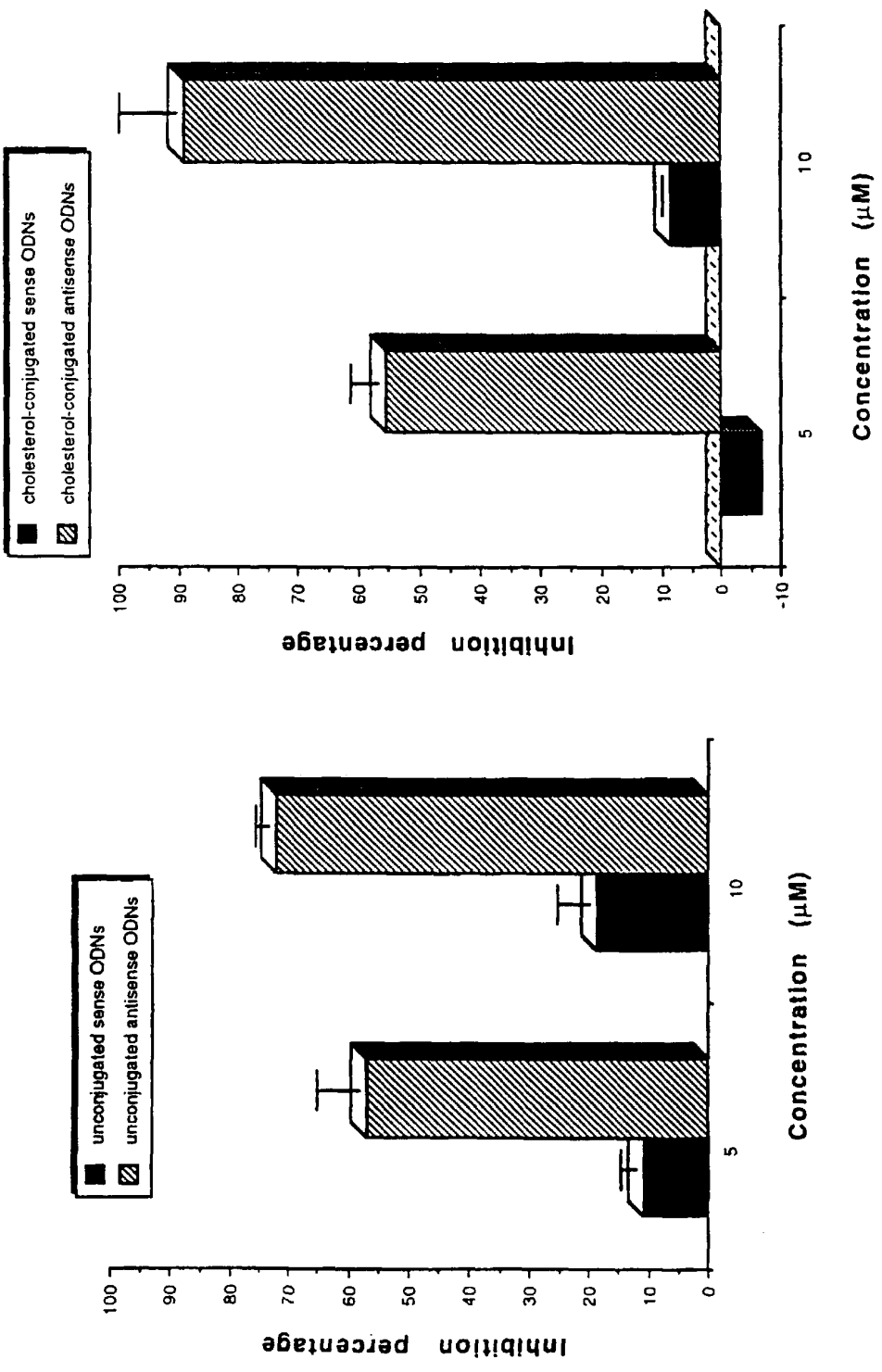

cholesterol-conjugated sense ODNs
cholesterol-conjugated antisense ODNs
Inhibition percentage
100
90
80
70
60
50
40
30
20
10
0
-10
5
10
Concentration (μM)
unconjugated sense ODNs
unconjugated antisense ODNs
Inhibition percentage
100
90
80
70
60
50
40
30
20
10
0
5
10
Concentration (μM)

FIGURE.3

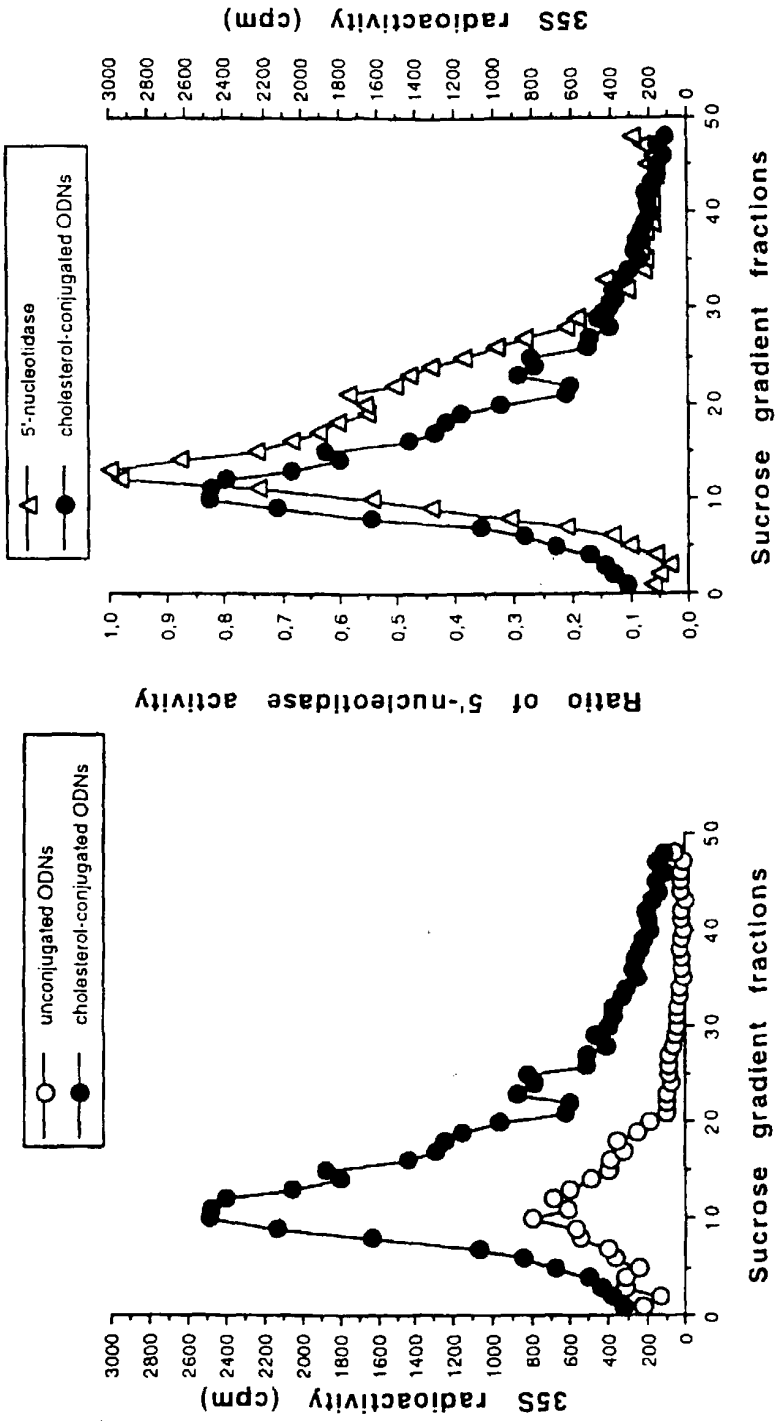

35S radioactivity (cpm)
Sucrose gradient fractions
unconjugated ODNs
cholesterol-conjugated ODNs
Ratio of 5'-nucleotidase activity
5'-nucleotidase
cholesterol-conjugated ODNs
35S radioactivity (cpm)
Sucrose gradient fractions

FIGURE 4

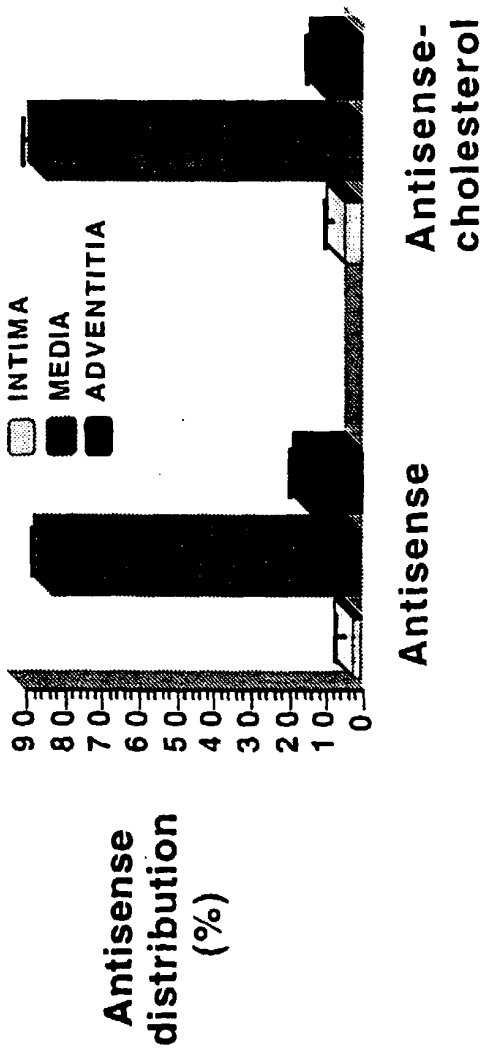

INTIMA
MEDIA
ADVENTITIA
Antisense
Antisense-cholesterol
Antisense distribution (%)
90
80
70
60
50
40
30
20
10
0

FIG. 5

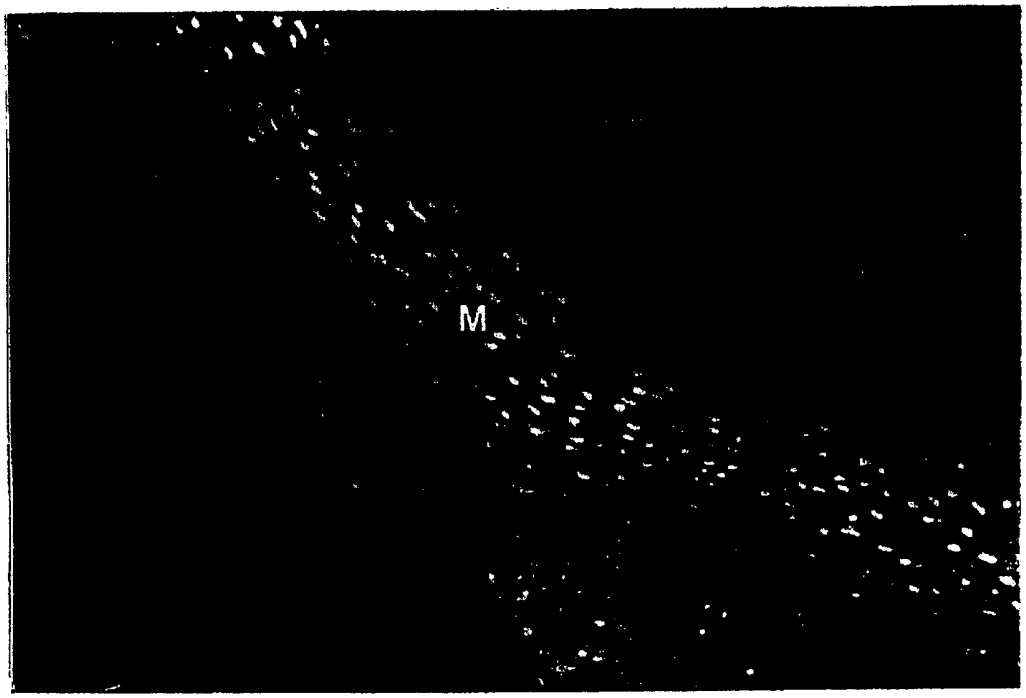


FIGURE 6

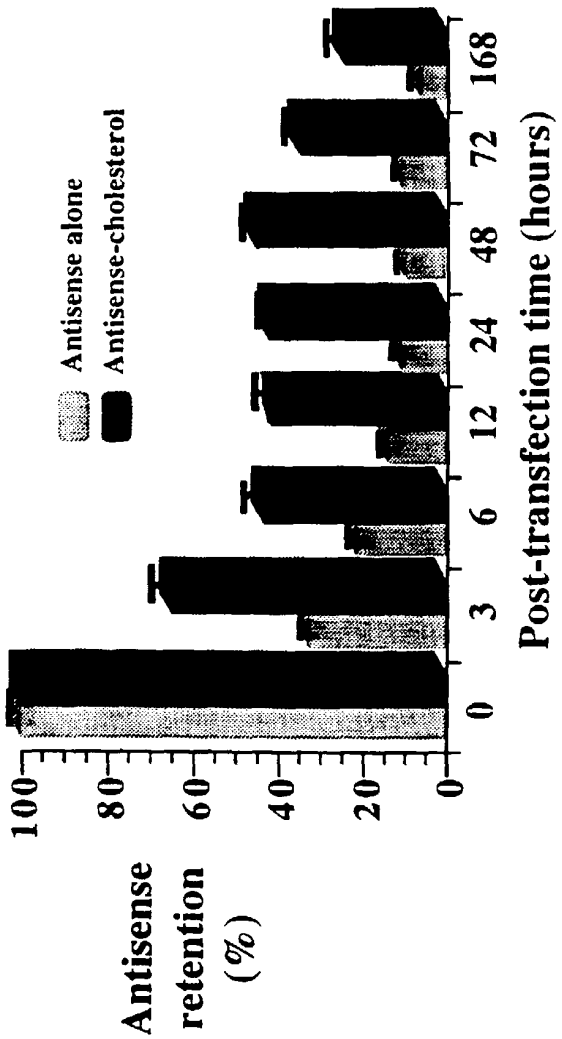

Antisense alone
Antisense-cholesterol
Antisense retention (%)
100
80
60
40
20
0
0
3
6
12
24
48
72
168
Post-transfection time (hours)

FIG. 7

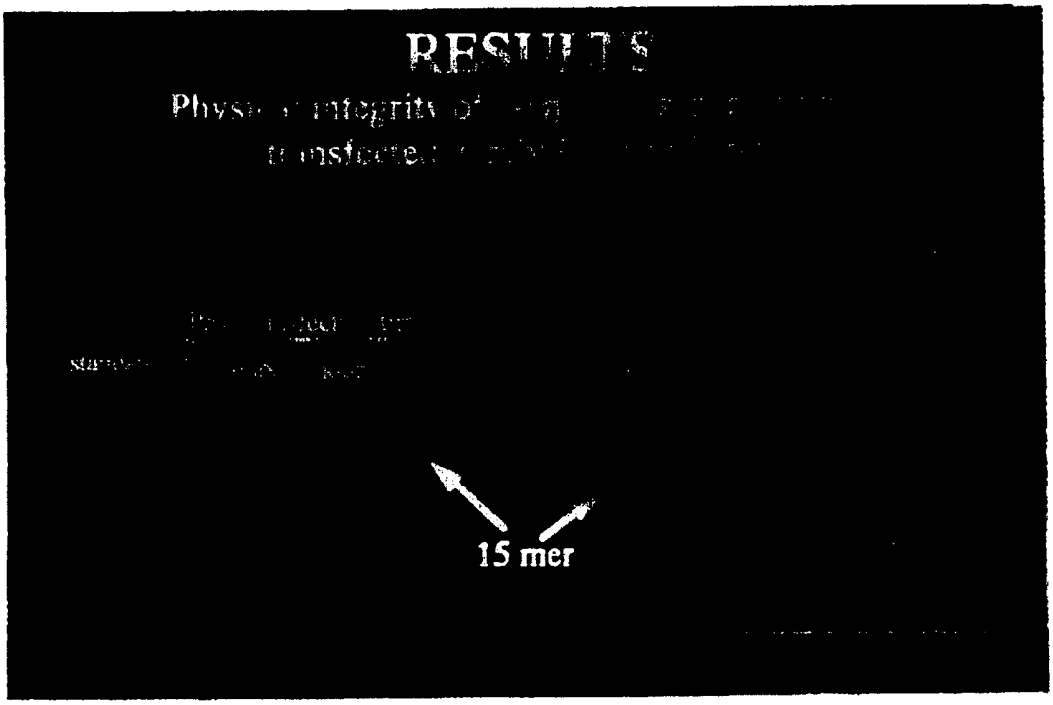


FIG. 8

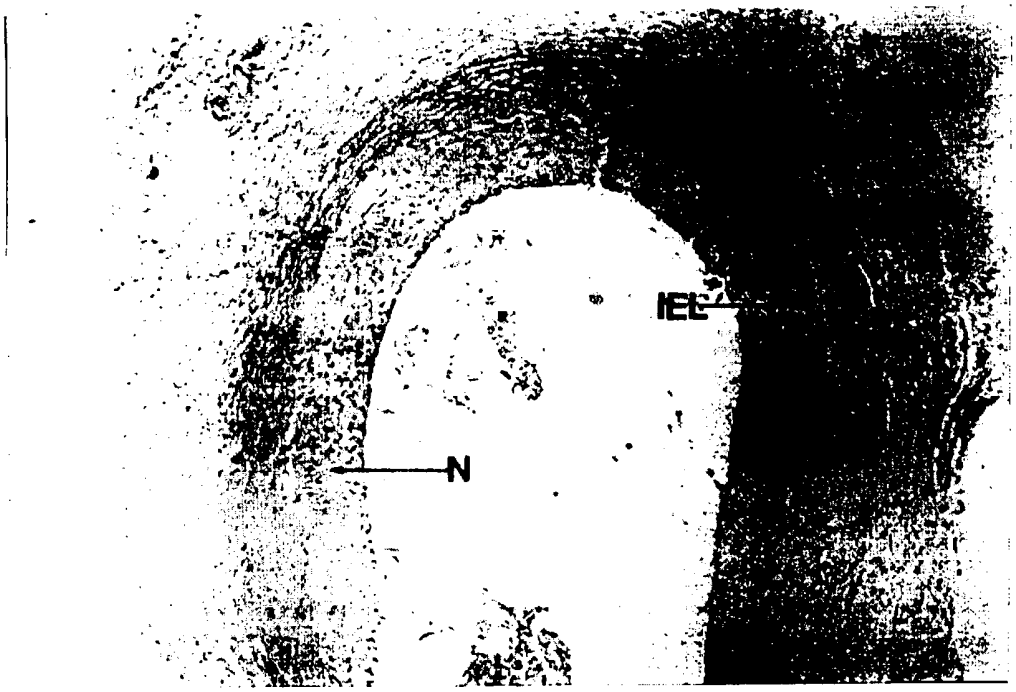


FIG. 9

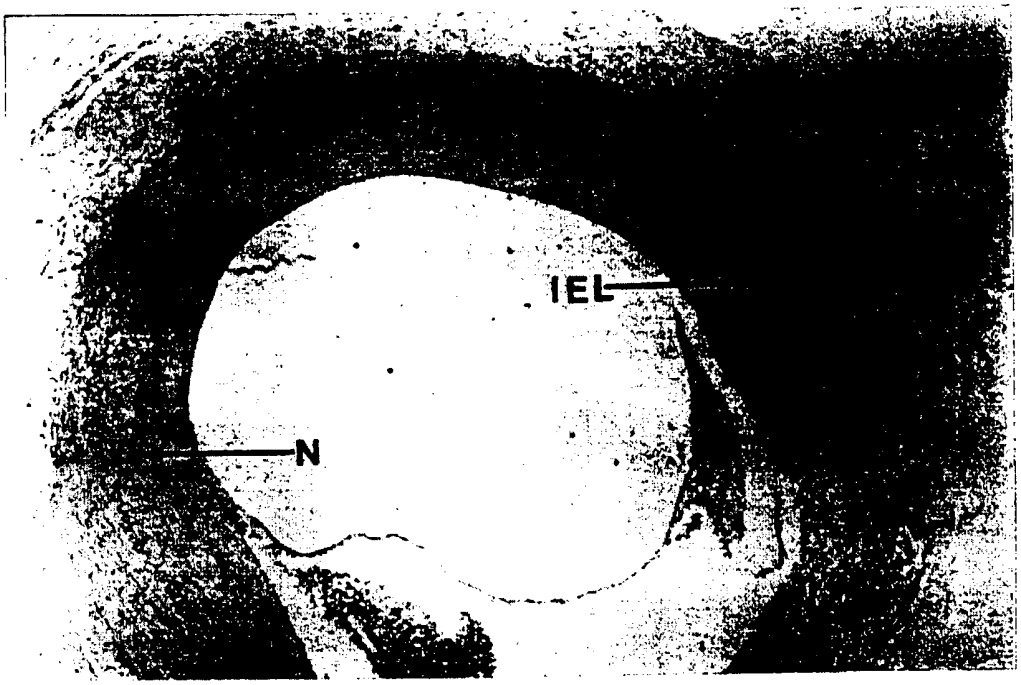


FIG. 10

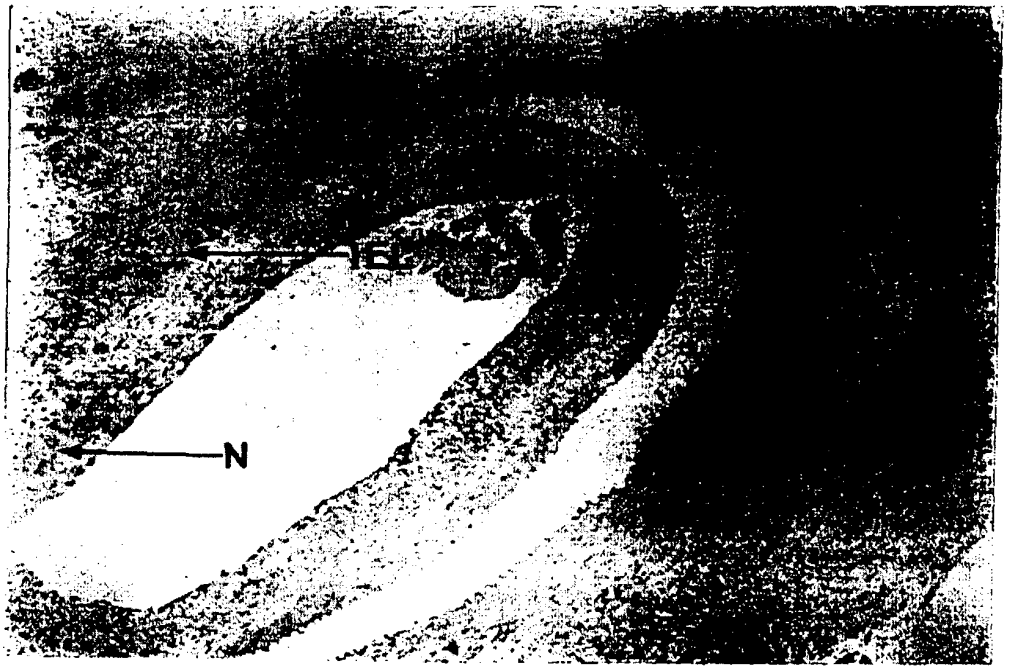


FIG. 11

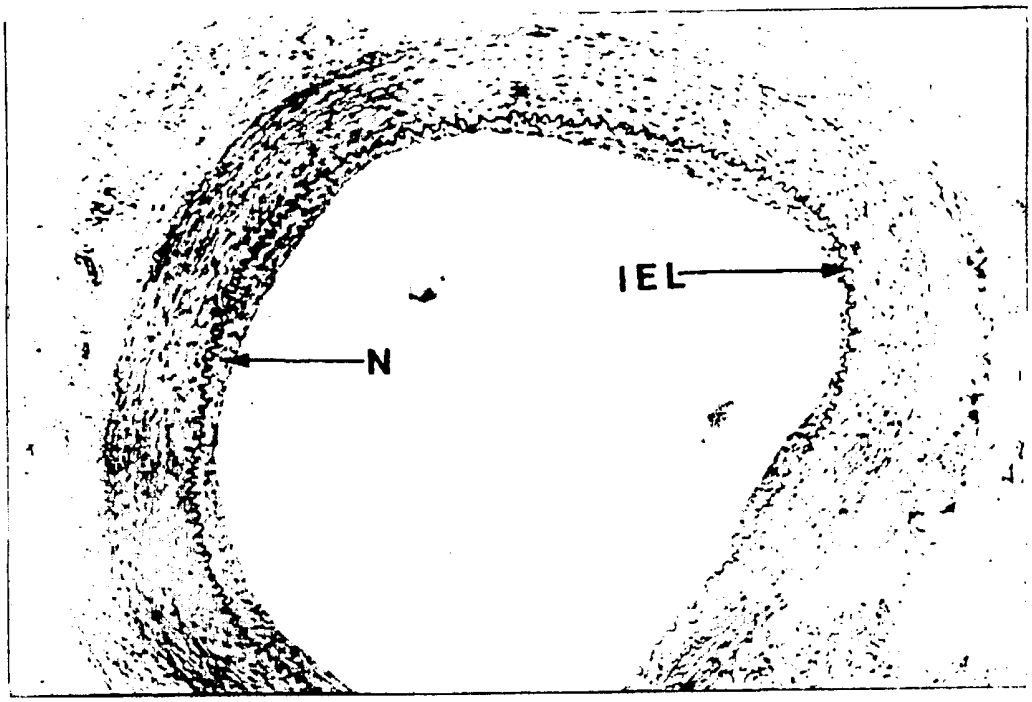
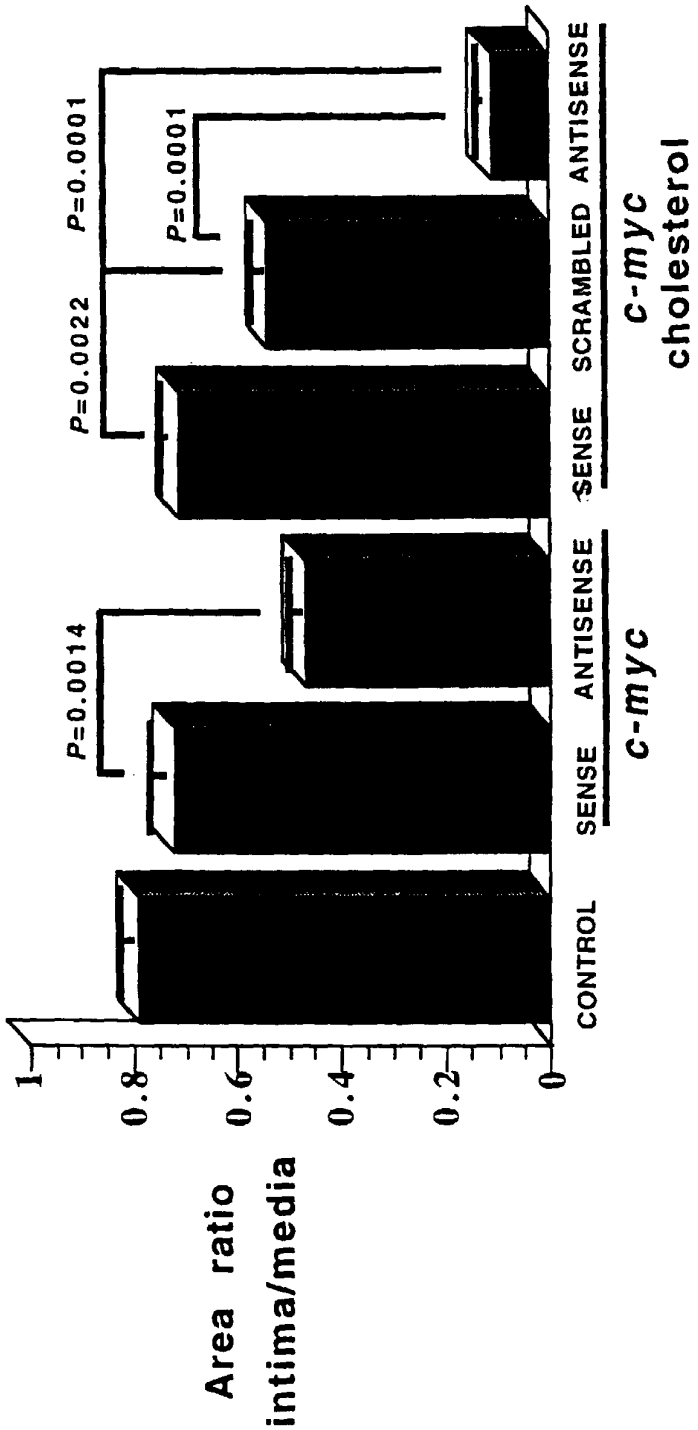


FIGURE 12


Area ratio intima/media
1
0.8
0.6
0.4
0.2
0
P=0.0014
P=0.0022
P=0.0001
P=0.0001
CONTROL
SENSE
ANTISENSE
c-myc
SENSE
SCRAMBLED
ANTISENSE
c-myc
cholesterol

INTERNATIONAL SEARCH REPORT

Inte...ational application No.
PCT/US96/16796

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 47/28, 48/00; C07H 21/02, 21/04; C12N 15/00

US CL :435/172.1, 240.1; 514/44; 536/23.1, 24.1, 24.5

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/172.1, 240.1; 514/44; 536/23.1, 24.1, 24.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, BIOSIS, SCISEARCH, CAPLUS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	BIRO et al. Inhibitory effects of antisense oligonucleotides targeting c-myc mRNA on smooth muscle cell proliferation and migration. Proc. Natl. Acad. Sci. USA. January 1993, Vol. 90, pages 654-658, see entire document.	1-6
Y	EBBECKE et al. Antiproliferative effects of a c-myc antisense oligonucleotide on human smooth muscle cells. Basic Res. Cardiol. 1992, Vol. 87, pages 585-591, see entire document.	1-6
Y	EDELMAN et al. c-myc in Vasculoproliferative Disease. Circulation Reasearch. February 1995, Vol. 76, No. 2, pages 176-182, see entire document.	1-6

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
03 JANUARY 1997	0 3 FEB 1997
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer SEAN MCGARRY Telephone No. (703) 308-0196

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INTERNATIONAL SEARCH REPORT

Inte...ational application No.
PCT/US96/16796

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	STULL et al. Antigene, Ribozyme and Aptamer Nucleic Acid Drugs: Progress and Prospects. Pharmaceutical Research. 1995, Vol.12, No. 4, pages 465-483, see esspecially Table VI.	1-6
Y	OBERHAUSER et al. Effective incorperation of 2'-O-methyl-oligoribonucleotides into liposomes and enhanced cell association through modification with thiocholesterol. Nucleic Acids Research. 1992, Vol. 20, No. 3, pages 533-538, see entire document.	1-6
Y	FLESER et al. Conjugation of C-Myc Antisense DNA with Cholesterol Significantly Increases In Vivo Oligomer Vascular Retention. Circulation. 15 October 1995, Vol. 92, No. 8, page I-296, Abstract number 1407.	1-6
Y	LETSINGER et al. Cholesteryl-conjugated oligonucleotides: synthesis, properties, and activity as inhibitors of replication of human immunodeficiency virus in cell culture. Proc. Natl. Acad. Sci. USA. September 1989, Vol. 86, pages 6553-6556, see entire document.	1-6
Y	BOUTORIN et al. Synthesis of alkylating oligonucleotide derivatives containing cholesterol or penazinium residues at their 3'-terminus and their interaction with DNA within mammalian cells. FEBS Letters. August 1989, Volumn 254, Number 1, 2, pages 129-132, see entire document.	1-6
Y	WICKSTROM et al. Longterm Antisense DNA Administration in C-MYC Transgenic Mice Bearing C-HA-RAS Induced Tumors. FASEB Journal. 1994, Vol. 8, No. 7, page A1448, abstract number 1102.	1-6
Y	SHI et al. Downregulation of c-myc Expression by Antisense Oligonucleotides Inhibits Proliferation of Human Smooth Muscle Cells. Circulation. September 1993, Vol. 88, No. 3, pages 1190-1195, see entire document.	1-6
Y	WO 94/23699 A1 (MEDISORB TECHNOLOGIES INTERNATIONAL L.P.) 27 October 1994, see entire document.	1-6
X, P --- Y, P	WO 96/24334 A1 (NEXSTAR PHARMACEUTICALS, INC.) 15 August 1996, see entire document.	1, 2, 5, 6 --- 3, 4

INTERNATIONAL SEARCH REPORT

Inte. ..ional application No.
PCT/US96/16796

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	WO 94/15646 A1 (THOMAS JEFFERSON UNIVERSITY) 21 July 1994, see entire document but especially page 11.	1, 2, 5, 6 ---------- 3, 4